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Depletion of Glycogen Synthetase and Increase of Glucose 6-Phosphate Dehydrogenase in Livers of Ethionine-Treated Mice

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1. Ethionine-treated mice showed a marked depletion in liver glycogen, a decrease of glycogen-synthetase activity, an increase in activity of glucose 6-phosphate dehydrogenase and the solubilization of phosphorylase. 2. The administration of cortisol or glucose did not alleviate these changes but the effect of ethionine was completely prevented in animals given methionine as well as ethionine. 3. The activities of the following enzymes were unchanged: hexokinase, glucokinase, glucose 6-phosphatase, phosphoglucomutase, 6-phosphogluconate dehydrogenase, UDP-glucose pyrophosphorylase, UDP-glucose dehydrogenase and pyruvate kinase.

The administration to rats of ethionine, the ethyl analogue of methionine, produces many characteristic biochemical lesions, among which are the development of fatty liver (Farber, Simpson & Tarver, 1950), depression of liver glycogen content (Lupu & Farber, 1954), inhibition of protein synthesis (Simpson, Farber & Tarver, 1950) and decrease of the ATP content of the liver (Shull, 1962).

The present paper reports some new findings in the liver of ethionine-treated mice: namely, the decrease in activity of glycogen synthetase (UDP-glucose–glycogen glycosyltransferase, EC 2.4.1.11), the elevation of the activity of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and the solubilization of phosphorylase (EC 2.4.1.1). The administration of cortisol or of glucose does not modify these phenomena, but the co-administration of methionine with ethionine does prevent them. A preliminary report on part of this work has been presented (Sie, 1963).

MATERIALS AND METHODS

Chemicals and enzymes. AMP, ADP, ATP, UDP-glucose (90% pure), pyruvate kinase (EC 2.7.1.40) type 1 (Sigma Chemical Co.), NAD^+, $NADP^+$, glucose 1-phosphate (dipotassium salt), glucose 6-phosphate (disodium salt), 6-phosphogluconate (tricyclohexylammonium salt), phosphoenolpyruvate (tricyclohexylammonium salt), DL-ethionine, rabbit-muscle phosphoglucomutase (EC 2.7.5.1) and yeast glucose 6-phosphate dehydrogenase, of highest purity, were obtained from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.; rabbit-liver glycogen was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; dehydroepiandrosterone and epiandrosterone were from Mann Research Laboratories Inc., New York, N.Y., U.S.A.; cortisol was a gift from the Upjohn Co., Kalamazoo, Mich., U.S.A.; pregnanolone was from General Biochemical Inc., Chagrin Falls, Ohio, U.S.A.; androst-4-ene-3,17-dione was from Dextran Chemicals Inc., New York, N.Y., U.S.A. All other chemicals were the best reagent grades available from commercial sources.

Animals. Three groups of 3-month-old male Ajax mice (25–30g.) were maintained on Purina Chow diet *ad libitum* specified according to three conditions: a control group (no dietary supplement), an ethionine group (dietary supplement, 0·5% of DL-ethionine), and an ethionine plus methionine group (dietary supplement, 0·5% of DL-ethionine plus 5·0% of DL-methionine). Each group consisted of six animals. Body weight and the amount of food intake were recorded daily for 4 days; then the animals were decapitated and bled and the livers removed quickly and chilled on ice.

To study the effects of cortisol, the animals were divided into two groups on the fourth day of ethionine feeding. At 9a.m., 0·1ml. of sterile distilled water was administered subcutaneously to one group, and 0·1ml. of cortisol suspension (1mg.) to the other. The animals were killed 4hr. after the injection.

Glycogenesis was attempted in the ethionine-fed animal by giving 0·6ml. (360mg.) of glucose solution to one group of animals by stomach tube and 0·6ml. of water to the control group. The animals were killed 6hr. after the glucose feeding.

Enzyme assay procedures. For phosphorylase, a solution of 0·25M-sucrose–0·1M-NaF–1mM-EDTA was used to prepare the 10% (w/v) tissue homogenates. For glucokinase (EC 2.7.1.2) assay, the whole liver was homogenized with twice its weight of a solution of 0·1M-tris–1mM-EDTA,

pH7·4. The other enzymes were assayed in 10% tissue preparations in 0·25M-sucrose–1mM-EDTA. The supernatant fractions were obtained after centrifugation of the homogenates at 100000*g* for 30min. at 0° in a refrigerated Spinco model L centrifuge.

All enzyme activities were determined in the supernatant fractions except for glucose 6-phosphatase (EC 3.1.3.9), which was determined in the homogenate, and glycogen synthetase and phosphorylase, which were measured in both homogenate and supernatant preparations. The particulate activity given represents the difference between the activities of homogenate and supernatant fractions.

The individual enzyme activity and the method employed for its measurement are indicated as follows: phosphoglucomutase (Najjar, 1948); soluble hexokinase (EC 2.7.1.1) (Crane & Sols, 1953); glucokinase (Viñuela, Salas & Sols, 1963); glucose 6-phosphatase (Cori & Cori, 1952; Fiske & Subbarow, 1925); phosphorylase (Cori, Illingworth & Keller, 1955; Leloir & Goldemberg, 1960); UDP-glucose dehydrogenase (EC 1.1.1.22) (Strominger, Maxwell & Kalckar, 1955); UDP-glucose pyrophosphorylase (EC 2.7.7.9) (Munch-Petersen, 1955); pyruvate kinase (Nigam, MacDonald & Cantero, 1962).

The estimation of glycogen synthetase, 6-phosphogluconate dehydrogenase (EC 1.1.1.44) and glucose 6-phosphate dehydrogenase are described in more detail as follows. Glycogen synthetase was assayed according to the method of Leloir & Goldemberg (1960). The reaction mixture consisted of 0·25μmole of UDP-glucose, 0·5μmole of glucose 6-phosphate, 7·5μmoles of glycine buffer, pH 8·5, 0·25μmole of EDTA, 0·4mg. of glycogen and 0·01ml. of enzyme, in a total volume of 0·05ml. The mixture was incubated for 15min. at 37°. The reaction was stopped by heating and UDP was estimated accordingly. 6-Phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase in the supernatant fraction were measured according to the method of Fitch, Hill & Chaikoff (1959) by following the rate of reduction of $NADP^+$ at 340mμ in a Beckman DU spectrophotometer. Two cuvettes of reaction mixture were prepared containing 200μmoles of glycylglycine buffer, pH 7·5, 4μmoles of $MgCl_2$, 0·025ml. of enzyme preparation, and 12μmoles each of glucose 6-phosphate and 6-phosphogluconate in one cuvette and 12μmoles of 6-phosphogluconate in the other. Then 2μmoles of $NADP^+$ were added last to start the reaction in a final volume of 1·0ml. A blank was set up similarly without the addition of substrate.

The specific activity of each enzyme is expressed as μmoles of substrate utilized or of product liberated/mg. of protein/hr. Protein was estimated according to the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine albumin serving as reference standard.

Other assays. Glycogen was extracted by hot KOH and precipitated by 95% (v/v) ethanol. It was estimated as glucose units by the cysteine–H_2SO_4 reaction (Dische, 1949).

Effect of steroids on partially purified glucose 6-phosphate dehydrogenase. Glucose 6-phosphate dehydrogenase free of 6-phosphogluconate-dehydrogenase activity was prepared from the livers of both control and ethionine-fed animals by fractionation with $(NH_4)_2SO_4$ (Glock & McLean, 1953). First, 50ml. of the supernatant fraction was brought to 70% saturation with respect to $(NH_4)_2SO_4$, the pH of the mixture being held at 7·0 by neutralizing with 3N-NH_3 solution in 70% saturated $(NH_4)_2SO_4$. The precipitate collected after centrifugation was extracted in successive solutions of 60%, 50%, 40% and 30% saturated $(NH_4)_2SO_4$. All the extracts were discarded except the one representing 30% saturation. Here the soluble protein was precipitated by adding solid $(NH_4)_2SO_4$ in an amount sufficient to make the saturation 40%. After centrifugation, the precipitate was dissolved in 2ml. of water and dialysed overnight against a large volume (5l.) of cold distilled water. The resulting enzyme preparation exhibited glucose 6-phosphate-dehydrogenase activity free of 6-phosphogluconate-dehydrogenase activity.

To study the specific inhibition of steroids on glucose 6-phosphate dehydrogenase, each steroid was dissolved in propylene glycol before studying its effect on glucose 6-phosphate dehydrogenase (Marks & Banks, 1960).

RESULTS

Table 1 indicates that there is little change in body weight in the control animals. However, there is a significant decrease in both food intake and body weight in groups of mice given ethionine, with or without methionine. These events did not affect the total liver weight of the animals in all three groups.

The ingestion of ethionine in mice produced a diverse effect on hepatic glycogen-synthetase and glucose 6-phosphate-dehydrogenase activities (Table 2). The former is profoundly decreased whereas the latter is significantly increased. These biochemical alterations are paralleled by a profound decrease in liver glycogen.

The activities of the glucose-phosphorylating enzymes, hexokinase and glucokinase, which have been distinguished from each other by their K_m values (Crane & Sols, 1953; Viñuela *et al.* 1963), were unchanged. No activity changes were noted for glucose 6-phosphatase, 6-phosphogluconate dehydrogenase, UDP-glucose pyrophosphorylase,

Table 1. *Effect of dietary ethionine on body weight, food consumption and liver weight*

Each group consisted of six animals.

	Control	Ethionine + methionine	Ethionine
Net change in body wt. (g.)	−0·1	−3·7±2·20	−4·6±1·60
Food intake (g./day)	4·2±0·73	1·8±0·83	2·5±0·90
Liver wt. (g.)	1·29±0·14	1·08±0·22	1·12±0·17

Table 2. *Effect of dietary ethionine on glycogen and on enzymes of the liver glycogen cycle*

Individual livers of six animals were assayed in each experimental group. For assay procedures see the text. Results are presented as means ± S.E.M. Enzyme activities are expressed as μmoles of product formed or substrate utilized/mg. of protein/hr.

	Control	Ethionine + methionine	Ethionine	Ethionine effect
Glycogen (g./100g.)	3·22±0·12	2·58±0·36	0·29±0·27	Decrease*
Specific enzyme activity				
Glycogen synthetase	1·15±0·06	1·18±0·05	0·34±0·03	Decrease*
Phosphorylase	4·80±0·70	4·82±0·52	5·54±0·25	0†
Glucose 6-phosphate dehydrogenase	0·31±0·06	0·39±0·03	1·15±0·10	Increase*
Hexokinase	0·20±0·07	0·22±0·10	0·22±0·06	0†
Glucokinase	0·36±0·07	0·35±0·08	0·39±0·09	0†
Glucose 6-phosphatase	2·04±0·09	1·98±0·04	1·81±0·08	0†
Phosphoglucomutase	78·4±4·90	88·2±6·3	65·2±3·80	0†
6-Phosphogluconate dehydrogenase	0·42±0·04	0·39±0·04	0·46±0·06	0†
UDP-glucose pyrophosphorylase	1·39±0·23	1·68±0·10	1·71±0·16	0†
UDP-glucose dehydrogenease	0·12±0·01	0·11±0·02	0·09±0·01	0†
Pyruvate kinase	1·59±0·11	1·45±0·09	1·75±0·08	0†

* Statistically significant ($P < 0·01$).
† No statistically significant change as compared with control values.

Table 3. *Effect of dietary ethionine on glycogen-synthetase and phosphorylase activities*

Individual livers of six animals were assayed in each experimental group. For assay procedures see the text. Results are presented as means ± S.E.M. Enzyme activities are expressed as μmoles of product formed or substrate utilized/mg. of protein/hr.

	Glycogen synthetase			Phosphorylase		
	Supernatant fraction	Particulate fraction	Total	Supernatant fraction	Particulate fraction	Total
Control	0·59±0·01	1·62±0·32	1·15±0·06	1·44±0·21	7·84±1·11	1·36±0·25
Ethionine + methionine	0·61±0·07	1·48±0·23	1·18±0·05	2·73±0·34	7·30±1·10	1·52±0·40
Ethionine	0·57±0·19	0·18±0·11	0·34±0·03	8·96±1·86	2·82±1·30	4·09±0·34

Table 4. *Glycogen content, glycogen synthetase, phosphorylase and glucose 6-phosphate dehydrogenase in the liver of the ethionine-fed mouse after cortisol or glucose administration*

Three animals in each group were investigated as described in the text. Enzyme activities are expressed as μmoles of substrate used or product formed/mg. of protein/hr.

Expt. no.	Treatment	Glycogen (g./100g.)	Specific enzyme activity: Glycogen synthetase in homogenate	Specific enzyme activity: Phosphorylase in supernatant (25000*g*)	Specific enzyme activity: Glucose 6-phosphate dehydrogenase in supernatant
1	Ethionine	0·02±0·01	0·05±0·04	13·3±1·52	1·66±0·47
	Ethionine + cortisol	0·04±0·01	0·21±0·08	11·0±1·66	1·65±0·11
2	Ethionine	0·02±0·00	0·43±0·02	8·70±1·30	1·69±0·41
	Ethionine + glucose	0·11±0·14	0·49±0·05	10·00±3·47	1·54±0·32

UDP-glucose dehydrogenase, phosphoglucomutase, pyruvate kinase and phosphorylase.

The particulate locations of glycogen synthetase and phosphorylase are shown in Table 3. Normally, the activities of these two enzymes were found to be associated with the particulate fraction (sedimentable at 100000*g* for 30min.). After the administration of ethionine, phosphorylase activity

Table 5. *Percentage inhibition of purified glucose 6-phosphate dehydrogenase by steroids*

Activities of the partially purified glucose 6-phosphate dehydrogenase preparations from normal and ethionine-fed mouse livers are 0·1 and 1·2μmoles/mg. of protein respectively/hr. The final steroid concentration in the 1ml. digest was 40μM. It was added in a solution of propylene glycol (0·01ml.). The control digest is identical but lacks the steroid in the 0·01ml. addition of propylene glycol. The conditions of assay were those of Fitch *et al.* (1959).

Inhibitor	Inhibition of glucose 6-phosphate dehydrogenase (%)	
	Normal mouse liver	Ethionine-fed mouse liver
Pregnanolone	51·7	57·8
Androst-4-ene-3,17-dione	39·3	35·0
Dehydroepiandrosterone	70·6	76·7
Epiandrosterone	80·9	84·7

was transferred from the particulate to the supernatant fraction ('solubilization'). Further, there was a decrease in glycogen synthetase in the particulate fraction, whereas its activity in the supernatant fraction remained unchanged. These abnormalities are not observed in animals receiving both ethionine and methionine.

In Table 4 the experimental results indicate that the administration of cortisol or of glucose to ethionine-fed animals failed to alleviate the biochemical changes induced by ethionine.

Finally, Table 5 shows no significant difference in the degree of inhibition by specific steroids of glucose 6-phosphate-dehydrogenase activity of preparations made from the livers of control and ethionine-fed animals.

DISCUSSION

Ethionine induces alterations in the intracellular distribution of glycogen synthetase and phosphorylase, which are normally associated with the glycogen pellet (Leloir & Goldemberg, 1960; Sie, Hablanian & Fishman, 1964). Phosphorylase was found to be 'solubilized' without any significant alteration in its activity, whereas glycogen synthetase was not solubilized but most of it disappeared from the particulate fraction. These events did not occur in preparations made from animals receiving both ethionine and methionine. Moreover, the parallel decrease in liver glycogen that can be produced by many other hepatotoxic agents, such as thioacetamide, carcinogenic azo dyes and carbon tetrachloride in rats (Spain & Griffith, 1957), puromycin (Hofert & Boutwell, 1963) and actinomycin D (Weber, Singhal & Stamm, 1963), may release enzymes of the glycogen particle into the cell cytoplasm. Tata (1964) postulated a simple physical binding between phosphorylase and the glycogen molecule to explain the solubilization of liver phosphorylase in starved rats. Shull (1962) observed a decrease of phosphorylase 5hr. after the administration of the ethionine to rats. Phosphorylase and glycogen synthetase do not necessarily fluctuate in the same way and the physical location of each enzyme may be contolled independently in the multi-enzyme glycogen particle.

The decrease in caloric intake does not appear to affect the liver weights of the experimental animals, indicating that toxicity cannot be a major factor in the explanation of the ethionine results.

Neither cortisol nor glucose was able to reverse the effects of ethionine, which are different from simple starvation, where glycogenesis could be induced by either cortisol or glucose (Sie & Fishman, 1964). The simultaneous intake of methionine with ethionine, however, prevents the occurrence of the biochemical aberrations. Among the several adaptive enzymes assayed under the present experimental conditions (Table 2), activity changes are observed for only two enzymes. The decrease of particulate glycogen synthetase has been discussed above. The other is the 300–500% elevation in the activity of glucose 6-phosphate dehydrogenase, whereas the activity of 6-phosphogluconate dehydrogenase is not significantly changed. With the concurrent administration of methionine and ethionine, no increase in glucose 6-phosphate-dehydrogenase activity is observed. Even though glucose 6-phosphate formation from phosphorylation of glucose by glucokinase has not been altered, the elevation of glucose 6-phosphate-dehydrogenase activity obviously would facilitate the removal of glucose 6-phosphate. This may result in a decrease in the cellular concentration of glucose 6-phosphate, which would diminish activation of glycogen synthetase in its catalysis of glycogen formation (Leloir & Goldemberg, 1960).

The question arose whether this increment in glucose 6-phosphate dehydrogenase was different in some respects from the enzyme present in the livers of control animals. A well-documented property of glucose 6-phosphate dehydrogenase from rat liver and adrenal gland, human liver and erythrocytes is its specific inhibition by the steroids dehydroepiandrosterone, epiandrosterone, pregnanolone and androst-4-ene-3,17-dione (Marks & Banks, 1960). In the present study, 6-phosphogluconate-dehydrogenase-free glucose 6-phosphate dehydrogenase preparations from both ethionine-fed and control animals were tested with these steroids and no significant difference in inhibition could be detected. Therefore, as far as sensitivity to

steroids is concerned, the glucose 6-phosphate dehydrogenase of ethionine-fed animals is indistinguishable from the glucose 6-phosphate dehydrogenase of normal animals.

The experimental finding of an increase in glucose 6-phosphate-dehydrogenase activity certainly is a noteworthy exception to the generalization that enzyme protein synthesis is inhibited by ethionine (Simpson *et al.* 1950). The hypothesis was proposed that the trapping of ATP in the form of *S*-adenosylethionine resulted in the general inhibition of energy metabolism in the hepatic cell (Stekol, Mody, Bedrak, Keller & Perry, 1960; Bartels & Hohorst, 1963). A decrease of hepatic ATP concentration in ethionine-fed rats was reported (Shull, 1962), and lower concentrations of NAD^+ and NADH were observed in the liver of both rats and mice (Stekol, Bedrak, Mody, Burnette & Somerville, 1962). It has been suggested that ethionine inhibits protein synthesis through an inhibition of messenger RNA synthesis (Villa-Trevino, Farber, Staehelin, Wettstein & Noll, 1964). Two recent reports, however, indicate a stimulation of RNA synthesis (Turner & Reid, 1964) and an increase in 'soluble' cysteine desulphydrase (Chatagner, 1964) in the rat after ethionine administration. The increase in glucose 6-phosphate-dehydrogenase activity may be connected with the former effect. Unless there is a specific activator, a loss of an inhibitor or a modification of the turnover rate of this specific enzyme protein, one would favour the existence of new synthesis of glucose 6-phosphate dehydrogenase.

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